

SUBSTRATE-ENZYME ORIENTATION DURING EMBRYONIC DEVELOPMENT¹

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INTRODUCTION

Since Godlewski's (1900) observations it has been known that the rate of respiration increases during the course of amphibian development. Numerous workers (cf. Moog, 1944) have confirmed these results with more accurate measurements. The work of Loeb (1895) and Philips (1940) on *Fundulus* embryos and Brachet (1934) on amphibians made it evident that a rigid causal dependence of embryogenesis on oxygen consumption does not obtain in all instances. These authors found that a considerable portion of early embryonic development could proceed in the complete absence of oxygen.

Relatively few attempts have been made to analyze experimentally the increase in oxygen consumption during development. Such an increase could presumably involve: (1) an increase in the permeability of the eggs to oxygen during development, (2) synthesis or activation of more enzymes, (3) formation of additional substrate, and (4) reorientation of enzyme and substrate, initially present, but functionally disconnected. This could be accomplished by a spatial separation between the two and the immobilization of a necessary carrier.

The first mechanism is made unpalatable by the findings of Parnas and Krasinska (1921), confirmed by Brachet (1934), who found the respiratory rate of amphibian embryos to be, within wide limits, independent of oxygen tension up to neurulation. During this same period a 4 to 5 fold increase in rate of oxygen consumption is realized.

It seemed probable that a systematic comparison of the respiration of brei and intact eggs during development combined with an examination of the cytochrome oxidase content could provide data which would permit a decision amongst the last three hypotheses mentioned. The present paper presents the results obtained from such experiments. They support the hypothesis that control of respiratory rate in developing embryos is effected by a spatial orientation of enzyme and substrate. No detectable increase in the cytochrome oxidase content was observed and the substrate content decreases rather than increases during development.

MATERIALS AND METHODS

Eggs

The eggs of *Rana pipiens* were obtained after injection of anterior pituitary gland. They were artificially fertilized and after swelling of the jelly were cut up

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into small groups in 10 per cent Ringers solution and kept at 20° C. The stages at which the eggs were selected for measurements were determined according to the schedules of Pollister and Moore (1937) and Shumway (1941). Where desirable intermediate stages were devised with the aid of photographs published in Rugh's (1941) manual.

In preparation for an experiment, the eggs were plucked clean of their jelly with the aid of a pair of fine forceps and filter paper. The denuded eggs were immediately placed in the M/15 phosphate buffer at pH 6.5. The number of eggs used in each flask varied at different stages. The attempt was made to use enough material to give ca. 5 mm. movement of the manometer fluid for each ten minute reading. The eggs were suspended in sufficient fluid so that the total volume was 2 cc. and transferred to the respirometer vessel.

Manometric measurements

All measurements were carried out at 26.2° C. in Barcroft-Warburg Manometric respirometers. The vessels were provided with side arms for substrate additions. The rate of shaking was 100 complete oscillations per minute with an amplitude of 8 cm.

Preparation of brei

After some preliminary experiments the following was adopted as the most satisfactory available method for obtaining breis of the eggs: The correct number of eggs was diluted to 20 cc. with chilled M/15 phosphate buffer and the suspension placed in a Waring blender. This volume is sufficient to almost cover the blades. When for various reasons less volume was used, tilting of the blender was resorted to during its operation. The blender was turned on intermittently until complete destruction of cell structure was attained as determined by direct microscopic observations. Care was taken to keep the temperature of the fluid below 25° C. during the process. Aliquots of the cell-free suspension were then dispensed to the vessels.

It may be noted that attempts to prepare cell-free breis by grinding with purified sand invariably led to considerable inactivation of the respiratory enzymes as evidenced by the consistently lower rates of oxygen consumption of such preparations as compared with those made with the blender. We had no success with the violent shaking procedure described by Brachet (1934). Fewer than 20 per cent of the embryos were disrupted by this method which in Brachet's hands yielded almost 100 per cent cytolysis. This difference may be attributed to a relatively greater sensitivity of the European species (*Rana temporaria*) to mechanical disruption.

The respiration of the breis starts to decline after about 60 minutes. Consequently rates were determined in the earlier constant rate portion. All values reported were obtained by measuring the slope of the straight line from 0 to 60 minutes.

Cytochrome oxidase

Cytochrome oxidase was measured following the precautions noted by Stotz (1939). In excess of 10⁻⁴ mM. per cc. of cytochrome C prepared by the method

of Keilin and Hartree (1937) from beef heart was included with each suspension in measuring cytochrome oxidase activity. As substrates, paraphenylenediamine (ppd) hydroquinone and adrenaline were employed in concentrations of 1/50th molar. In the case of the latter two, a correction for autooxidation was necessary. This was done by including one flask in which all reagents were used as before except that the cell extract was placed in boiling water for 5 minutes. The readings so obtained were subtracted from those of the other flasks. Such corrected readings did not differ from those obtained with paraphenylenediamine which has a negligible rate of autooxidation. Care was taken to keep the tissue content per flask below levels which would yield respiratory rates exceeding 600 cu. mm. per hour when substrate was added.

Within 30 minutes of the addition of substrate from the side arm, a very sharp drop in the rate of oxygen consumption is observed. Five minute interval readings were taken after the introduction of substrate and the calculation of cytochrome oxidase activity is based on the slope of the straight obtained by plotting the oxygen consumption against time in the 10–20 minute period following the addition of substrate. All the cytochrome oxidase activity measurements on the breis were performed within 60 minutes of their preparation.

EXPERIMENTAL RESULTS

Using the methods described, a detailed comparison was made of the respiratory rates of breis and whole eggs during the course of development. The results obtained are summarized by Figure 1.³ The general exponential nature of the curve describing the variation of respiratory rates of intact embryos during development agrees with those recently published by previous authors (Atlas, 1938; and Moog, 1944). An exception may be noted in the data reported by Barnes (1944) in which a linear rather than exponential rate of rise is observed between stages 12 and 17. Barnes does not discuss this discrepancy between her results and those published earlier by Atlas.

It may be noted in passing that in agreement with Atlas (1938) and Moog (1944) the curve for intact cells in Figure 1 does not rise continuously. A break occurs between stages 12 and 13, a little later in development than the discontinuities observed by the other authors.

The respiration of the brei is high from the very earliest stages of development and remains so well into stage 13 which corresponds to the onset of neurulation. Beyond this stage however it starts to fall, remaining however above the rate of intact eggs in corresponding stages until about stage 17. Beyond stage 18 the respiration rates of the breis are definitely below those of the corresponding embryos. These results confirm the observations of Brachet (1934) who noted that cytolysis of the eggs of *Rana temporaria* during early embryogenesis led to marked increases in the rates of oxygen consumption whereas later in development decreases always followed cytolysis.

It is quite apparent from the respiratory rates of the breis that the eggs contain from the very onset of development sufficient enzymes and oxidizable substrate to support a much higher rate of oxygen uptake than the intact embryos actually do.

³ The authors are deeply indebted to Mrs. Helen Spiegelman for her able assistance in making the many calculations.

From this result alone it would seem unnecessary to postulate the formation of additional enzyme or substrate to account for the rising respiratory rate during development. The early high rates of the breis can be explained by assuming that the destruction of the cellular structure permits a freer contact of substrate with enzyme than occurs in the normal embryo. The fall in respiration of the brei observed beyond stage 13 can also be explained on the same basis. With the passage of time more and more substrate is consumed resulting in a depression of the brei respiratory rate due to substrate, or possibly carrier, dilution.

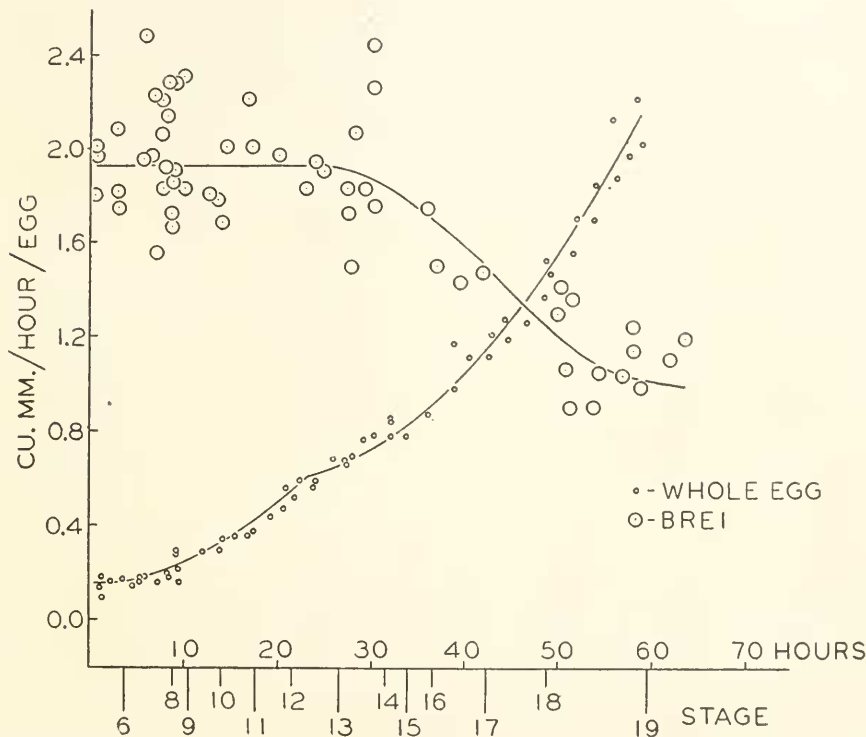


FIGURE 1. Comparison of respiration rates of brei and whole embryo (egg) during the course of development.

Response to cytochrome C

While it was not expected that externally placed carriers could penetrate into the cells, experiments were performed in which very heavy concentrations of cytochrome C, up to 2×10^{-3} mM. per cc., were placed in the external media in which embryos in early stages were suspended. No effect on respiratory rate could be detected.

Since the fall in brei respiration beyond stage 13 might possibly be due to a decrease through loss or inactivation of cytochrome C, this substance was added to breis prepared at various stages of development. Control readings were taken for

approximately $\frac{1}{2}$ hour before transferring sufficient cytochrome C from the side arms to the main compartment to give a concentration of at least 10^{-4} mM. per cc. This concentration was chosen since it gave maximum rates when substrates of the cytochrome oxidase system were also added. The results are summarized in Table I. It is evident from this table that no significant changes occur on adding cytochrome C. Thus brei respiration is not limited by the content of this factor and the fall in rate beyond stage 13 cannot be ascribed to variations in its concentration.

TABLE I

The effect on respiration rate of adding cytochrome C to brei prepared at different stages of development. All values are based on the cu. mm. of O_2 consumed/egg/hour.

Stage	Respiration before addition of cyt. C	Respiration after addition of cyt. C	Change
3	1.91	1.90	-0.01
5	2.20	2.10	-0.10
6	1.85	1.90	-0.05
7	2.04	2.04	0.00
8	2.13	2.21	+0.08
9	1.92	1.92	0.00
10	1.81	1.87	+0.06
11	2.00	2.00	0.00
12	1.92	1.81	-0.11
13	2.06	2.00	+0.06
14	1.62	1.62	0.00
15	1.72	1.70	-0.02
16	1.41	1.30	-0.11
17	1.22	1.22	0.00
18	0.93	1.02	+0.09
19	1.15	1.20	+0.05

Response to addition of cytochrome oxidase substrates

Paraphenylenediamine or hydroquinone was added in the presence of excess cytochrome C to breis prepared from different stages. Figure 2 summarizes the data obtained. The respiration level following the addition of substrate is, under the conditions of these experiments, proportional to the cytochrome oxidase activity. It is clear from the results that this activity is, as far as can be determined, constant throughout development. Consequently neither the rise in the respiratory rates of the intact embryos nor the fall in the respiratory rates of the breis observed in the later stages can be ascribed to variations in the content of this enzyme. The data obtained with added substrates are consistent with the view that the burst of respiration following cytolysis is due to the breakdown of controls, exerted by geometrical constraint, on existent substrates and enzymes.

It is worthy of note that the addition of the cytochrome oxidase substrates raises the respiration at all stages considerably above that attained by the brei alone. This might indicate that even in the early stages all the available active centers of the enzyme are not completely saturated by endogenous substrate when a brei is prepared.

The results of adding the cytochrome oxidase substrates with or without excess cytochrome C to intact eggs at different developmental stages are summarized in

Table II. For purposes of comparison the same table includes data on the rates of breis following similar additions. For the most part these breis were prepared from the same groups from which individuals were used for the intact embryo experiments.

It is clear that in comparison with the marked increases obtained with the breis, the addition of substrate has relatively little effect on the respiration of intact embryos. No consistent strong increase is obtained at any time from stage 3 up to and including stage 19.

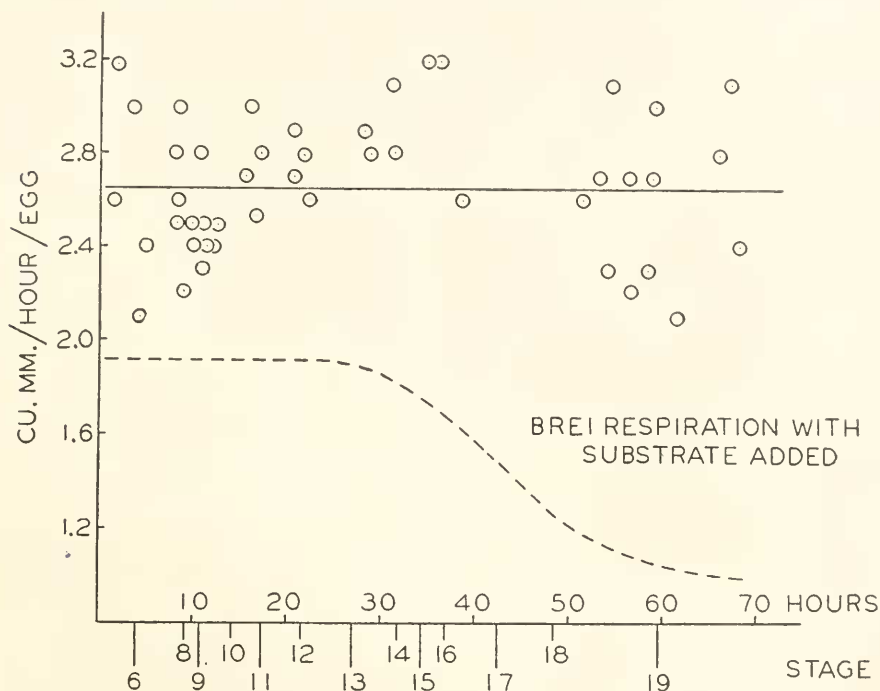


FIGURE 2. The effect on respiration rate of adding substrates of cytochrome oxidase to brei during development. The dotted line represents the curve taken from Figure 1. Single points represent individual experiments.

It is impossible to explain these results on an inability of these substances to penetrate the cells since all of the agents are effective in stopping further development and cause a severe bleaching of the melanin pigment. Both of these facts indicate with some certainty that substrates do penetrate the cells. Thus a simple lack of substrate does not limit the rate of respiration of the intact cells. Carrier immobilization would appear to be the most plausible explanation, although it is conceivable that endogenous substrate somehow forms a protective shell around active centers.

EXPERIMENTS WITH INHIBITORS

Assuming that the respiration is largely mediated through the cytochrome oxidase system, it was of some interest to examine the action of inhibitors of cyto-

chrome oxidase. According to Keilin and Hartree (1939) both cyanide and azide are effective against this enzyme.

Response of brei and intact embryos to NaCN

In these experiments 0.001 M NaCN was used since it was found by preliminary experiments that higher concentrations did not give significantly greater depressions. The usual precautions suggested by Krebs (1935) were observed and appropriate mixtures of alkali and cyanide were used in the center wells. The results

TABLE II

Respiration (cu. mm./egg/hour) of brei and whole eggs on addition of cytochrome oxidase substrate. Substrate concentration M/50; cytochrome C 10^{-4} mM.

Stage	Whole egg	Brei	Whole egg substrate	Brei substrate	Change on adding substrate	
					Whole egg	Brei
3	0.21	1.6	0.20	3.2	-0.01	+1.6
	—	1.7	—	2.6	—	+0.9
	—	2.1	—	3.0	—	+0.9
4	0.21	—	0.20	—	-0.01	—
	0.20	—	0.20	—	0.00	—
	0.25	—	0.21	—	-0.04	—
5	0.17	1.8	0.26	2.1	+0.09	+0.3
	0.15	—	0.16	—	+0.01	—
	—	1.8	—	2.4	—	+0.4
7	—	2.2	—	2.2	—	0.0
	0.20	2.2	0.40	3.0	+0.20	+0.8
	0.15	2.2	0.20	2.5	+0.05	+0.3
	—	2.2	—	2.6	—	+0.4
	0.23	—	0.24	—	+0.01	—
	0.26	1.9	0.21	3.0	-0.05	+1.1
8	—	1.7	—	2.4	—	+0.7
	0.27	2.3	0.29	2.3	+0.02	0.0
	0.30	2.4	0.31	2.4	+0.01	0.0
8 ³⁺	0.18	1.7	0.26	2.8	+0.08	+1.1
	0.20	2.1	0.22	2.4	+0.02	+0.3
	0.20	—	0.23	—	+0.03	—
8 ⁸⁺	—	1.7	—	2.5	—	+0.8
8 ⁹⁺	—	2.3	—	2.5	—	+0.2
8 ¹²⁺	0.35	—	0.32	—	-0.03	—
	—	1.9	—	2.5	—	+0.6
10	0.40	2.1	0.60	3.0	+0.20	+0.9
	0.34	2.2	0.30	2.8	-0.06	+0.6
	—	2.0	—	2.7	—	+0.7

TABLE II—*Continued*

Stage	Whole egg	Brei	Whole egg substrate	Brei substrate	Change on adding substrate	
					Whole egg	Brei
12	0.45	1.9	0.50	2.6	+0.05	+0.7
	0.58	1.7	0.58	2.8	0.00	+1.1
	—	1.8	—	2.7	—	+0.9
	0.50	2.2	0.53	2.9	+0.03	+0.7
12 ⁸⁺	0.57	—	0.70	—	+0.13	—
	0.60	—	0.56	—	+0.06	—
13	0.67	2.4	0.67	3.1	0.00	+0.7
	0.64	2.6	0.64	2.9	0.00	+0.3
	0.65	2.9	0.65	2.8	0.00	-0.1
13 ⁴⁺	0.78	—	0.78	—	0.00	—
	0.75	—	0.63	—	-0.12	—
14	0.96	1.3	0.87	2.8	-0.09	+1.5
	—	1.6	—	3.1	—	+1.5
16 ¹⁺	1.1	1.3	0.83	3.2	-0.18	+1.9
	—	1.2	—	3.2	—	+2.0
	1.2	—	0.94	—	-0.26	—
16 ³⁺	—	1.3	—	2.5	—	+1.2
18 ¹⁺	—	0.78	—	2.2	—	+2.1
18 ²⁺	1.7	1.4	1.8	2.6	+0.1	+1.2
18 ³⁺	2.1	1.0	2.2	3.1	+0.1	+2.1
	2.1	0.90	2.6	2.9	+0.5	+2.0
18 ⁵⁺	1.6	0.98	1.2	2.3	-0.4	+1.3
	—	0.89	—	2.7	—	+1.8
18 ⁸⁺	—	0.89	—	2.7	—	+1.4
18 ¹²⁺	1.9	0.97	1.6	2.1	-0.3	+1.1
	2.1	0.90	1.8	2.7	-0.3	+1.8
	—	1.00	—	2.3	—	+1.3
19	2.4	1.0	2.8	2.4	+0.4	+1.4
	2.6	0.97	2.0	2.8	-0.6	+1.83
	—	0.85	—	3.1	—	+2.25

obtained are summarized in Figure 3. The data show that a strong inhibition of whole egg respiration is obtained at all stages. The respiration of the brei, while not diminished to levels quite as low, is also strongly depressed. Figure 4 gives the trend of cyanide sensitivity in terms of per cent inhibition and is based on calculations of average values from Figures 1 and 3. The fall in sensitivity of the brei in

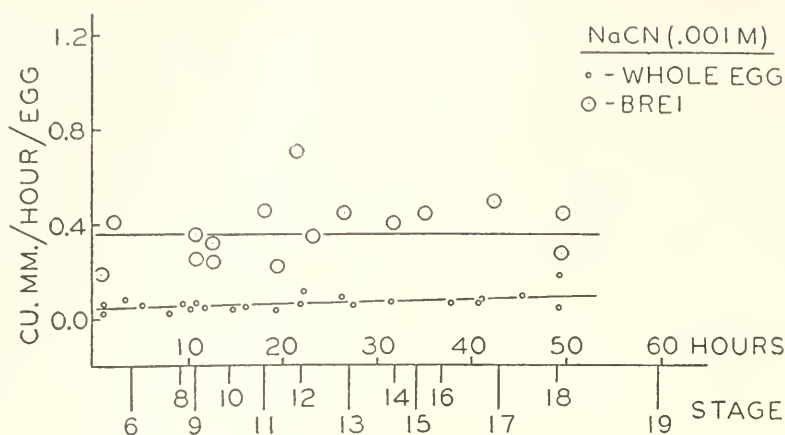


FIGURE 3. Respiratory rate in the presence of cyanide of brei and intact embryos during development.

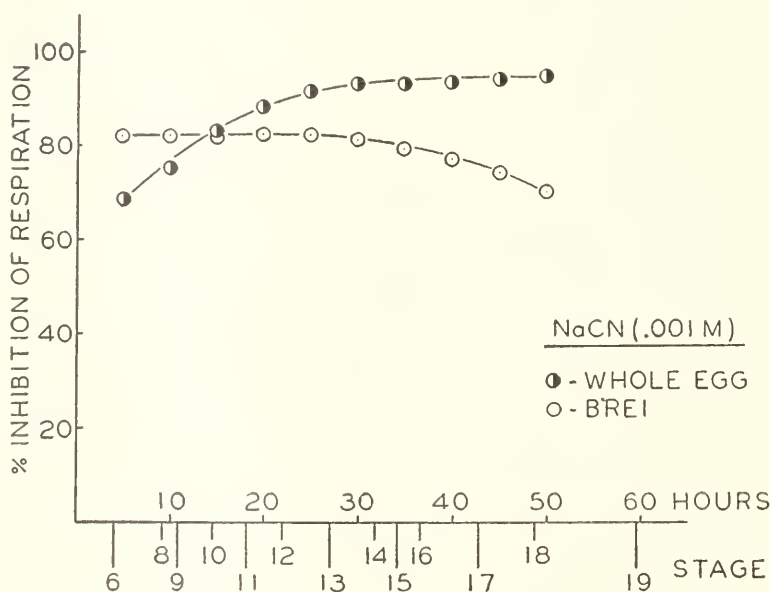


FIGURE 4. Cyanide sensitivity of intact embryo and brei respiration during development.

the later stages is a numerical consequence of the diminishing normal rate during this period which has been attributed to substrate limitations.

These data show that a major portion of the respiration of both brei and intact eggs is mediated via a cyanide sensitive system and that the increments in respiratory rate observed with development are due to the gradual augmentation in the activity of this cyanide sensitive system.

Response of intact embryos and brei to sodium azide

The same type of experiments as were carried out with NaCN were also performed with sodium azide at pH 6.7. The concentration selected was 0.005 M since higher concentrations up to and including 0.1 M did not result in more pronounced effect. The respiratory rates of intact embryos and corresponding breis obtained in the presence of azide are given in Figure 5. The percentage inhibitions at the various developmental stages calculated on the basis of averages from Figures 1 and 5 are shown in Figure 6.

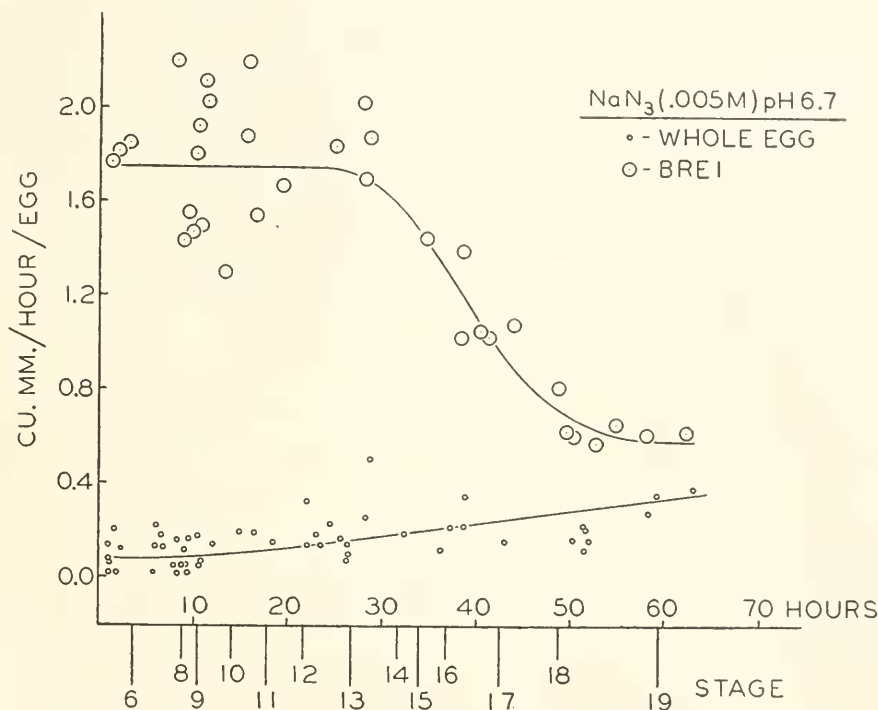


FIGURE 5. Respiratory rates in the presence of azide of breis and intact embryos during development.

In general (Figs. 5 and 6) the behavior of intact embryos towards azide is not very different from that with cyanide. However the residual azide insensitive respiration is consistently greater than its cyanide sensitive counterpart throughout development. Furthermore, unlike the cyanide insensitive respiration which remains constant, the absolute value of the azide insensitive respiration rises during development.

The actions of cyanide and azide on brei are strikingly different. Up until stage 13, 0.005 M azide can decrease the respiration of brei by only 10 per cent. It should be emphasized that this relative ineffectiveness of azide as a respiratory inhibitor against brei is not confined to this concentration only. As may be seen from column 2 of Table III it is true for concentrations as high as 0.1 M.

While Keilin and Hartree (1939) came to the conclusion that azide was virtually equivalent to cyanide in inhibiting the cytochrome oxidase system, recent experiments (Winzler, 1943; Starnard, 1939; Armstrong and Fischer, 1940) show that this conclusion is not universally applicable.

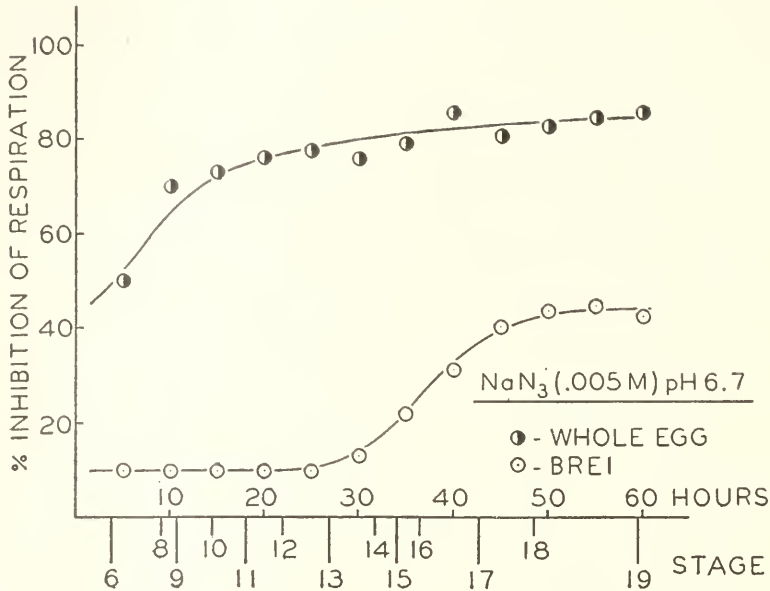


FIGURE 6. Azide sensitivity of intact embryo and brei respiration during development.

To test whether azide attacks the cytochrome oxidase in the breis in the concentrations employed as well as at higher ones, breis were prepared from embryos in stage 9 and placed in the main compartment of Warburg vessels with adequate amounts of cytochrome C and various concentrations of NaN_3 . Sufficient para-

TABLE III

Effect of various concentrations of NaN_3 on O_2 consumption of brei when paraphenylenediamine is added. Rate is cu. mm./egg/hour. All eggs in stage 9.

M conc. of azide	Rate before substrate addition	Rate after substrate addition	Δ
0.000	1.91	2.51	+0.60
0.005	1.70	2.29	+0.59
0.01	1.80	2.50	+0.70
0.05	1.63	1.75	+0.13
0.10	1.65	1.67	+0.02

phenylenediamine was placed in the side arms to yield a final concentration of 1/50th molar. After an adequate number of readings were taken to establish the rate without substrate, the substrate was introduced and the resulting respiration followed.

The results are given in Table III. Concentrations up to and including 0.01 M are unable to prevent the rise which occurs on adding substrate. The two higher concentrations tested, 0.05 and 0.1 molar, although ineffective against the respiration of the endogenous substrate, were capable of preventing the burst which normally follows the addition of paraphenylenediamine.

Similar experiments were carried out with cyanide (Table IV). Cyanide not only depresses the brei respiration to levels previously noted but in addition prevents any rise when the paraphenylenediamine is added.

TABLE IV

Effect of various concentrations of NaCN on O₂ consumption of brei when paraphenylenediamine is added. Rate is cu./mm./egg/hour. All eggs stage 9.

M conc. of NaCN	Substrate rate before addition	Rate after substrate addition	Δ
0.000	2.14	2.86	+0.72
0.001	0.51	0.51	0.00
0.002	0.32	0.38	+0.06
0.003	0.24	0.24	0.00
0.005	0.44	0.54	+0.10

These experiments with cyanide establish with some degree of certainty that the normal brei respiration is mediated by the Warburg-Keilin system. At least three important criteria for this assertion are satisfied: (1) The system exists in the brei. (2) The response to cytochrome oxidase substrate is cyanide sensitive. (3) The respiration of endogenous substrate is also cyanide sensitive. It is therefore necessary to assume that insensitivity of the brei respiration to azide is due to the latter's inability to combine with the cytochrome oxidase in the brei. It is conceivable that azide cannot combine readily with the cytochrome oxidase when the other enzymatic components and substrates of the cell are present in the extract.

It is certainly reasonable to expect that the sensitivity of a given enzyme system to a particular inhibitor will depend on the kind and amount of the substrate with which it is competing for active centers. It is therefore not surprising that cytochrome oxidase should be more sensitive to azide when in comparative isolation. That some such competitive mechanism is working may be seen from an analysis of the data in Table III and in particular from the fact that the two highest concentrations employed, while leaving the respiration of endogenous substrate relatively untouched, effectively suppress the increased oxidation when paraphenylenediamine is added. These findings would be expected if at the higher concentrations of azide the inhibitor could compete successfully for an active site on the enzyme surface with a molecule of paraphenylenediamine but not with a molecule of endogenous substrate. Further, it will be recalled (see Fig. 6) that azide sensitivity of the brei respiration starts to rise beyond stage 13 when the normal rate starts to fall due to substrate limitations. This finding would also be expected, since with substrate concentration falling the ratio of azide to endogenous substrate rises and the azide can then start to compete successfully for active centers.

While the above interpretation fits all the data obtained on the relative azide insensitivity of brei it leaves unexplained the effective inhibitory action on intact embryos shown in Figure 5. If we interpret this inhibition as a reaction between the Warburg-Keilin system and azide we would necessarily conclude that this agent can combine with cytochrome oxidase in intact eggs but not in cell extracts. A plausible reason for this difference may be seen by focussing attention on substrate availability in the two situations. Here again we probably have a situation analogous to the increasing azide sensitivity of brei when the rate is falling i.e., less severe competition by endogenous substrate molecules for active centers as their concentration decreases. It is undoubtedly true that in the intact eggs the number of substrate molecules getting to the enzyme surface is severely regulated, permitting the azide molecules to compete successfully.

It is possible to explain the different responses of brei and whole egg to azide by assuming that in neither case can the azide combine with cytochrome oxidase in the concentrations employed. The capacity of azide to inhibit synthetic processes has been demonstrated for certain systems (Winzler et al., 1944; Moog and Spiegelman, 1942). Most important for the present discussion is the finding by Spiegelman and Moog (1945) that azide completely inhibits amphibian development at all stages including those between fertilization and gastrulation. From this point of view then, azide may depress the respiratory rates of intact eggs indirectly because it inhibits synthetic activities leading to substrate availability for enzyme action.

DISCUSSION

The data presented find their most plausible interpretations in terms of structure orientation and the consequence of its destruction. The comparison of brei and intact embryos in the course of development as well as the determination of cytochrome oxidase content ruled heavily against enzyme or substrate synthesis as explanations for the rising respiratory rate during development. This emphasis on the internal geometry of enzyme and substrate and its variation as determining factors is a point of view that is becoming increasingly popular. Recent reviews by Korr (1939) and Commoner (1942) have stressed it as a criterion for evaluating data obtained from *in vitro* experiments. Both Runnstrom (1930) and Korr (1937) have discussed the sudden activation of the Warburg-Keilin system on fertilization of *Arbacia* eggs in terms of the relative positions of enzyme and substrate. Ballentine (1940a, b) who studied the dehydrogenase activity in the same material suggested similar considerations for these enzyme systems. It seems inescapable that more refined interpretations of physiological processes must consider not only what enzymes and substrates exist in the cell, but also where they exist. The present investigation which explains rising respiratory rate in terms of reorientation of existent components indicates that this is equally true for embryonic processes.

SUMMARY

The respiratory rates of whole embryos and cell-free breis were determined at various stages of development. During early stages, when the respiration of whole

embryos is low, brei respiratory rate is high, the situation being reversed beyond about stage 17.

Cytochrome oxidase remains uniformly high throughout development as shown by the effects of adding substrate to breis. Externally applied cytochrome oxidase substrates have little effect on intact embryos although the agents are shown to penetrate. Cytochrome C is shown not to be a limiting factor in brei respiration.

Cyanide depresses the respiration of both breis and embryos. Azide has little effect on respiration of breis but is very effective in depressing oxygen consumption and development of intact embryos.

The results are discussed in terms of spatial orientation of enzymes and substrates.

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